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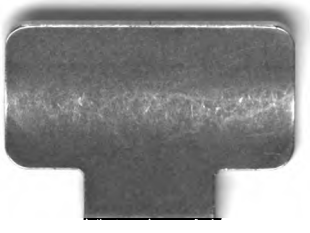

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BULLETIN No. 233. JUNE, 1903.

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GENEVA, N. Y.

RENNET-ENZYME AS A FACTOR IN CHEESE-RIPENING.

L. L. VAN SLYKE, H. A. HARDING AND E. B. HART.

PUBLISHED BY THE STATION.

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BULLETIN No. 233.

RENNET-ENZYME AS A FACTOR IN CHEESE-RIPENING.

L. L. VAN SLYKE, H. A. HARDING AND E. B. HART.

SUMMARY.

I. The object of the work described in this bulletin was to ascertain to what extent the formation of soluble nitrogen compounds in cheese-ripening is due to the rennet-extract used in cheese-making. In the case of the work previously done here and elsewhere, the effect of rennet-enzyme has not been studied apart from the action of other factors that are present in cheese-ripening. It was our purpose to study its action by itself, apart from other proteolytic agents.

II. The action of rennet-extract was first studied in cheese containing rennet-enzyme as the only proteolytic factor, with and without acid, and also with and without salt. In these experiments (44 to 51), all milk-enzymes were destroyed by heating the milk at 95°C. to 98°C. (203°F. to 208°F.), the coagulable property of the milk-casein was restored by the addition of either calcium chloride or carbon dioxide gas, and all organisms were rendered inactive by chloroform. Acid, when present, was furnished by addition of pure lactic acid.

III. The action of fresh rennet-extract on casein in milk, with and without acid, was studied in comparison with old rennet-extract, and also in comparison with commercial pepsin. In these experiments, the milk-enzymes were

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destroyed by heat and all organisms were rendered inactive by chloroform.

IV. The action of rennet-extract in cheese was studied in comparison with commercial pepsin. In these experiments, (55 to 57), the milk-enzymes were destroyed by heat and commercial pepsin was added in different amounts. No chloroform was used and there were present, therefore, such organisms as were introduced during the process of making cheese. Acid was furnished by addition of hydrochloric acid.

V. The action of rennet-extract on paracasein dilactate was studied in comparison with commercial pepsin. In these experiments, rennet-enzyme and commercial pepsin, sterilized by formaldehyde, were allowed to act upon sterile paracasein dilactate.

VI. The action of rennet-extract was studied in cheese containing acid-forming and some proteolytic organisms. In these experiments (52 and 53), the milk-enzymes were destroyed by heat, acid was furnished by a lactic-acid "starter," but no chloroform was used. We thus had as our only proteolytic agents rennet-enzyme in the presence of acid and such organisms as were introduced in the "starter" or that got into the milk or curd during the operation of cheese-making.

VII. Special work was done to show that all milk-enzymes were destroyed by heat. Bacteriological examinations were made of the cheese and milk.

VIII. In the case of every experiment made, there was little or no digesting action by either rennet-enzyme or commercial pepsin in the absence of acid, while the action was marked in the presence of acid.

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IX. In the absence of acid in cheese, no paracasein lactate is found and little or no proteolysis occurs; in the presence of acid in the cheese, paracasein monolactate is formed and digestion takes place, the rennet-ferment being the active agent. The ability of rennet-enzyme to convert paracasein into soluble nitrogen compounds appears to depend upon the presence of acid, resulting in the formation of paracasein monolactate.

X. Rennet-enzyme and commercial pepsin act essentially alike in forming soluble nitrogen compounds, when compared with each other in the case of cheese, milk and paracasein dilactate.

XI. In the case of both rennet-enzyme and commercial pepsin, the chemical work performed by the ferments is confined mainly to the formation of the paranuclein, caseoses and peptones, while only small amounts of amides are formed, and no ammonia.

XII. Rennet-enzyme is really a peptic ferment.

XIII. Salt, in the proportions found in normal cheese, appears to have little effect upon the action of rennet-enzyme in cheese-ripening. The experiments on this point are, however, not regarded as conclusive.

XIV. The abnormal conditions present in many of the experiments, such as pasteurized milk, calcium chloride and chloroform, would tend, if they had any effect at all, to decrease the digestive action of rennet-enzyme. Our results, therefore, may properly be regarded as representing the minimum effect of rennet-enzyme in cheese-ripening.

XV. The digestive action of rennet-enzyme does not appear to extend to the formation of compounds that produce the flavor of cheese.

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INTRODUCTION.

In Bulletin No. 203 of this Station, we published the results of some preliminary work, in which we made a study of the relation of the enzymes contained in milk to the ripening process of cheese. We aimed to exclude bacterial action in cheese and thus limit our study to the results produced by the enzymes present in the milk when made into cheese, including rennet-enzyme. In our previous work, we made no attempt to distinguish between the different enzymes in respect to their individual action in cheese-ripening. The object of the work described in this bulletin was, primarily, to ascertain to what extent the proteolytic phenomena of cheese-ripening are due to the action of an enzyme contained in the rennet-extract used in cheese-making.

It has been quite generally believed that the rennet-extracts used in the manufacture of cheese contain not less than two enzymes or ferments, called rennin and pepsin, one ferment coagulating milk-casein and the other converting milk-casein and paracasein, under favorable conditions, into soluble forms of nitrogen compounds. The present tendency, however, is in the direction of the belief that both kinds of action are due to the presence of only one enzyme. The presence of a proteolytic ferment in rennet-extract is readily understood, when we consider its source, which is the stomach of a suckling calf.

For years the weight of opinion was against the belief that rennet has any other function in cheese-making than simply to coagulate milk-casein. In Bulletin No. 54, page 267, the results of some experiments made at this Station in 1892 are given, and it was shown that cheese made with larger amounts of rennet furnished greater quantities of soluble nitrogen compounds than did cheese made with smaller amounts of rennet. In 1899 some additional work was done, confirming the results previously obtained. Babcock, Russell and Vivian[1] have made a very thorough investigation of this subject, showing that, in the case of normal cheese, increased use of rennet resulted in a more rapid increase of soluble nitrogen compounds, especially

[1] Annual Report. Wis. Exp. Sta., **17** : 102 (1900).

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of those nitrogen compounds grouped under the names of caseoses and peptones. They also made cheese from milk to which purified commercial pepsin had been added and found similar chemical changes taking place in the cheese thus made. They concluded from these experiments with normal cheese that rennet exerts a digestive influence on casein, due to the presence of peptic enzymes contained in rennet-extracts, the action of which is intensified by the development of acid in the cheese-curd. Jensen,[2] working independently and along quite different lines, reached the same conclusions at the same time.

In the case of the work previously done here and elsewhere, the effect of rennet-ferment has not been studied apart from the action of other factors that are present in normal cheese-ripening. So far as our present knowledge goes, the different agencies taking part in the normal process of cheese-ripening are the following: (1) Some acid, usually lactic; (2) enzymes present in the milk before it is made into cheese; (3) an enzyme contained in the rennet-extract added to milk in the cheese-making process; and (4) micro-organisms, chiefly bacteria. In previous studies of the effect of rennet-ferment on cheese-ripening, some or all of these factors have been present, so that the specific action of rennet has had to be inferred rather than been clearly proved. It has been the special aim of our work to study the action of the rennet-ferment as far as possible apart from the other agencies of cheese-ripening. Under these conditions, we have studied the action of rennet-extracts in cheese-ripening,—(1) without acid, (2) in the presence of acid, (3) without salt, and (4) with salt. In addition, we have studied the action of rennet-extracts of different ages upon the casein of milk, and also the proteolytic action of commercial pepsin on milk-casein and in the process of cheese-ripening. We have also studied the action of rennet-enzyme and pepsin on paracasein dilactate.

DESCRIPTION OF EXPERIMENTAL WORK.

DIFFICULTIES INVOLVED IN THE WORK.

In order to destroy all enzymes present in milk, our general plan of procedure has been to heat the milk to a temperature

[2] *Landw. Jarhb. d. Schweiz.*, **14**: 197 (1900).

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varying in different cases from 85° C. to 98° C. (185° F. to 208° F.). Then, in order to prevent possible contamination by the entrance of enzyme-producing organisms, the milk, after being heated and cooled, has been treated with 3 to 5 per ct. of chloroform by volume, previous to being made into cheese. The heating of milk to the temperature stated diminishes the readiness and completeness with which it is coagulated by rennet-extract, but the power of prompt coagulation by rennet can be restored by the addition of calcium chloride or carbon dioxide or any ordinary acid. In thus eliminating other factors of cheese-ripening than rennet-enzyme, we necessarily produce conditions that do not exist in normal cheese-making, such as (1) heated milk, (2) absence of milk-enzymes, (3) the use of calcium chloride or carbon dioxide, and (4) absence of enzyme-forming and acid-forming organisms. In a study carried on under such conditions, we cannot expect our results to be entirely comparable with results obtained under normal conditions; but we can secure data that enable us to determine the ability of the rennet-enzyme to cause proteolytic changes under the conditions of experiment employed. Later, we will inquire as to whether the introduction of such unusual conditions seriously affected the value of the results obtained, in their application to the process of normal cheese-ripening.

GENERAL OUTLINE OF EXPERIMENTAL WORK.

For each cheese made, we used from 40 to 75 pounds of normal milk, making a cheese adapted in size to the most convenient conditions of our work. Chloroform, when used, was introduced into the milk as soon as the milk had been heated to 85° C. to 98° C. (185° F. to 208° F.) and cooled to 29° C. (84° F.). The process of making cheese was then carried out in the usual manner. At the time of adding chloroform, samples of milk were taken out and carefully kept for chemical and bacteriological examinations, in order to ascertain whether any proteolytic enzymes remained active.

In experiments 44 to 47, calcium chloride was added to the milk to restore its coagulable power with rennet. In doing this, a solution was made containing 200 grams of pure calcium

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chloride in 500 cc. of water, and we used 2.5 cc. of this solution for each kilogram of milk. Carbon dioxide was used in place of calcium chloride in experiments 48 to 51. In using this, we passed a vigorous stream of the gas through the milk for about 30 minutes previous to adding rennet. After several trials, we found that calcium chloride and carbon dioxide, used in the manner described, enabled the rennet-extract to coagulate the milk completely in 20 to 30 minutes. Hansen's rennet-extract was used at the rate of 2.5 liquid ounces for 1,000 pounds of milk (about 1 part of rennet-extract to 600 parts of milk by weight). In those experiments in which we compared the effect of the presence and absence of salt, a double portion of milk was generally used and the operation of cheese-making was carried on as usual to the point of salting, when the curd was divided into two approximately equal parts, one portion not being salted and the other portion receiving salt at the rate of 2 pounds of salt for 1,000 pounds of milk.

The cheeses were taken from the press and at once put under air-tight vessels in an atmosphere of chloroform, where they were kept during the period covered by our study. For additional details regarding the use of chloroform in cheese-making and cheese-ripening, see Bulletin No. 203, page 327.

The first series of experiments included 44 to 47. In these calcium chloride was used to restore the coagulability of the milk. Lactic acid was added in 45 and 46 and omitted in the others. Salt was added in 46 and 47 and omitted in 44 and 45. In all cases the milk was heated and treated with chloroform.

After a month, in was noticecd that there was little indication of proteolytic change, and it was thought possible that the presence of calcium chloride might retard the action of the rennet-enzyme. It was then decided to repeat the experiments, using carbon dioxide in place of calcium chloride, and this second series included experiments 48 to 51.

In experiments 52 and 53, the milk was pasteurized at 85° C. (185° F.), carbon dioxide was added and the acid was furnished by a "starter," as in normal cheese-making. No chloroform was used. In 53 salt was used and omitted in 52.

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In experiments 55, 56 and 57, the milk was pasteurized at 85° C. (185° F.) and cheese made with and without the use of commercial pepsin.

The details of the conditions of the individual experiments and of the results of chemical analysis are fully given in the Appendix.

THE RELATION OF RENNET-ENZYME TO CHEESE-RIPENING IN THE ABSENCE OF ACID.

In experiments 44, 47, 49 and 50, no acid was added and the conditions of experiment prevented the formation of acid, except possibly in minute quantities before the milk was heated. While the conditions of these experiments differ in some details, they were all as nearly alike as possible in respect to the absence of acid. Table I contains the results of chemical analysis when the cheese was fresh from press and when 12 months old.

TABLE I.—SHOWING EFFECT OF RENNET-ENZYME IN CHEESE-RIPENING IN ABSENCE OF ACID.

No. of experiment.	Age of cheese when analyzed.	Nitrogen, expressed as percentage of nitrogen in cheese, in form of—			
		Water-soluble nitrogen compounds.	Paracasein monolactate.	Paranuclein, caseoses and peptones.	Amides.
		Per ct.	*Per ct.*	*Per ct.*	*Per ct.*
43	Fresh.	4.67	2.46	3 22	1.45
43	12 mos.	9.70	3 33	5.61	4.09
44	Fresh.	1.93	2.44	1.93	0
44	12 mos.	6.25	2 72	2.96	3.29
47	Fresh.	3.67	2.90	3.67	0
47	12 mos.	6.41	3.46	3.08	3.33
49	Fresh.	10.95	5.24	9.92	1.03
49	12 mos.	10 34	4.27	5.16	5.18
50	Fresh.	10.12	5.72	8.57	1.55
50	12 mos.	9.67	3.02	5.74	3.93

If we compare the amount of water-soluble nitrogen compounds found in the fresh cheese and at the end of one year, we see readily that there was little or no advance in the proteolysis taking place in this period of time. It is also significant that there was little, if any, paracasein monolactate formed. The results of these experiments indicate that the rennet-ferment, in

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the absence of acid, does little or no work in the formation of soluble nitrogen compounds in the process of cheese-ripening.

In passing, it may be well to speak of the sources of the soluble nitrogen compounds found in fresh cheese, that is, cheese about 24 hours old. The milk-albumin is a fairly constant source of soluble nitrogen. This is retained in cheese as a constituent of the whey, and the quantity retained depends largely upon the amount of whey held in the cheese. In ordinary normal cheese, the amount varies from 1.2 to 1.5 per ct. of the nitrogen in the cheese, but in extreme cases may exceed 2 per ct. In addition to milk-albumin, we have, as a source of soluble nitrogen compounds in fresh cheese, slight amounts of proteolytic products formed from casein and paracasein during the operation of cheese-making. The amount from this source varies with the conditions of manufacture. It is probable that paracasein and paracasein monolactate are slightly soluble in water and may contribute small amounts to the soluble nitrogen compounds of the fresh cheese.

In the cheeses used in most of the experiments described in this bulletin, excessive amounts of whey were unavoidably retained in the cheese, and the soluble nitrogen compounds found in the fresh cheese are therefore larger than in cheese holding less moisture.

ACTION OF RENNET-ENZYME IN THE PRESENCE OF ACIDS IN CHEESE-RIPENING.

In experiments 45, 46, 48 and 51, we added to the milk enough lactic acid to equal about 0.2 per ct. of the milk by weight. We thus had only two factors that could act as proteolytic agents in the cheese, the rennet-enzyme and the acid. The results of these experiments are given in Table II.

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TABLE II.—SHOWING THE EFFECT OF RENNET-ENZYME IN THE PRESENCE OF ACID IN CHEESE-RIPENING.

No. of experiment	Age of cheese when analyzed.	Nitrogen, expressed as percentage of nitrogen in cheese, in form of —			
		Water-soluble nitrogen compounds.	Paracasein monolactate.	Paranuclein, caseoses and peptones.	Amides.
		Per ct.	*Per ct.*	*Per ct.*	*Per ct.*
45	Fresh	4.65	27.88	4.65	0
45	12 mos.	18.50	9.80	14.38	4.12
46	Fresh	5.40	26.62	3.96	1.44
46	12 mos.	18.50	11.97	14.02	4.48
48	Fresh	4.26	29.80	3.41	0.85
48	15 mos.	47.06	11.76	40.00	7.06
51	Fresh	3.89	22.92	3.10	0.79
51	15 mos.	19.32	12.83	15.06	4.26

In studying the data contained in Table II, we notice the following results:

(1) In every instance there was an increase of water-soluble nitrogen compounds. In most of the cases the increase was about one-third or one-fourth of what we find in a normal cheese, excepting No. 48, in which the amount was much nearer the results given by normal cheese. The increase in this case was probably due in part to the fact that during the first few weeks of ripening, this cheese was placed in a temperature of 21° C. (70° F.), while the others were kept at 15.5° C. (60° F.). It was probably still more due to the larger amount of moisture carried by 48, which was 10 to 15 per ct. greater than in 51.

(2) The increase of soluble nitrogen compounds was confined largely to the paranuclein, caseoses and peptones, the amount of amides remaining small. In normal cheese-ripening, we find these relations reversed, that is, the amides form a considerably larger part of the soluble nitrogen compounds than do the higher groups.

(3) In all of the cheeses, when fresh, we had a considerable and fairly uniform amount of paracasein monolactate, which compound was practically absent in the cheese containing no acid.

(4) The results embodied in Tables I and II may properly be interpreted as showing that the proteolytic action of the rennet-

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enzyme in cheese-ripening is dependent upon the presence of acid.

ACTION OF RENNET-EXTRACTS OF DIFFERENT AGES AND OF COMMERCIAL PEPSIN ON MILK-CASEIN.

In considering the results obtained in cheese-ripening by the use of rennet-extract, the question may arise as to whether the observed proteolytic changes were due to rennet-enzyme alone or whether the rennet may not have contained some proteolytic bacterial enzymes produced in the rennet-extract previous to its use. In order to answer this question, we tried the effect of two samples of rennet-extract upon milk-casein, using one extract known to be in good condition and one known to be old and apparently in the first stages of putrefaction. These experiments were carried out in the following manner: We heated 8.6 liters of milk for 15 minutes at 85° C. (185° F.), and after cooling added 2 per ct. of chloroform by volume. Of this milk, we placed in each of several bottles 100 cc. In one case, we added to the neutral milk 0.22 cc. of Hansen's fresh rennet-extract, and in another the same amount of old rennet-extract. In other bottles, we added, in addition to the rennet-extract, 0.5 cc. of pure concentrated lactic acid. For comparison, we placed in other bottles, with and without acid, the same amount of milk and 0.06 gram of Parke, Davis & Co.'s aseptic scale pepsin for each 7 grams of proteid contained in the milk. Duplicates were used in all cases. The contents of these bottles were kept at 15.5° C. (60° F.) and were examined at intervals both chemically and bacteriologically. With the exception of a single determination in the case of one bottle, the germ content was below 50 per cc., which undoubtedly represented spore forms.

The results of chemical analysis are given in the subjoined table.

The determinations of nitrogen in the form of amides were made by the use of phosphotungstic acid, since it has been shown[3] that, in the case of peptic digestion, phosphotungstic acid is a more satisfactory reagent than tannic acid, especially in solutions having an acid reaction. The amount of nitrogen originally in the milk was 0.561 per ct.

[3] New York Agr. Exp. Sta. Bul. No. 215, pp. 90 and 98 (1902)

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TABLE III.—SHOWING THE ACTION OF RENNET-EXTRACTS OF DIFFERENT AGES ON MILK-CASEIN.

Kind of rennet extract used.	With or without lactic acid.	Age of milk when analyzed.	Nitrogen, expressed as percentage of nitrogen in milk, in form of —		
			Soluble nitrogen compounds.	Caseoses and peptones.	Amides.
			Per ct.	*Per ct.*	*Per ct.*
.....	.....	Fresh	9.98	.....	.....
Fresh.....	Without	1 mo.....	11.35	6.00	5.35
"	With	" "	29.80	22.22	7.58
Old.....	Without	" "	12.57	4.99	7.58
"	With	" "	25.23	18.55	6.68
Fresh.....	Without	3 "	15.86	7.17	8.69
"	With	" "	41.89	31.73	10.16
Old.....	Without	" "	17.02	8.95	8 07
"	With	" "	36.45	26.29	10.16
Fresh.....	Without	6 "	17.49	14.82	2.67
"	With	" "	47.15	41.17	5 98
Old.....	Without	" "	14.40	10.65	3 75
"	With	" "	40 83	35.74	5 09
Fresh.....	Without	9 "	18.98	13.63	5.35
"	With	" "	53.57	45.64	7 93
Old.....	Without	" "	17.03	12.13	4.90
"	With	" "	47.96	39.67	8.29
Commercial pepsin	Without	1 "	8 91	2.22	6.69
"	With	" "	33 51	25.93	7.58
"	Without	3 "	11.42	2.42	9.00
"	With	" "	44.47	34.22	10.25
"	Without	6 "	10.34	6.60	3 74
"	With	" "	48.76	44.74	4.02
"	Without	9 "	10.08	6.51	3 57
"	With	" "	56.96	48.05	8 91

The data embodied in Table III appear to be quite definite in respect to the following points:

(1) At any given time, the fresh rennet-extract had, in most cases, formed a larger amount of soluble nitrogen compounds than had the old extract. This was particularly true in acid solution. This result does not indicate that we had bacterial enzymes in the old rennet in addition to rennet-enzyme. The difference in action of the two rennet-extracts is not marked in the class of amido compounds. If the old extract contained bacterial enzymes, we should expect it to produce larger amounts of amido compounds. These results fail to show that the old

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rennet-extract contained any proteolytic bacterial enzymes, as compared with the fresh extract.

(2) If we compare the results secured by the use of the purest commercial pepsin with those given by the rennet-extracts, we find that, in the presence of acid, there are formed soluble nitrogen compounds quite close in amount to those formed by rennet-extract. The amount of soluble nitrogen compounds formed in neutral solution was fairly stationary during the 9 months, while, in the case of the rennet-extracts, there was a slow increase. The amount of amido compounds was surprisingly uniform in the case of the pepsin and the rennet-extracts, in both neutral and acid reaction. These results suggest that the pepsin was able to account for all the changes observed in the case of the rennet-extracts in the presence of acid. If there had been proteolytic enzymes of bacterial origin in the rennet-extracts, we should have expected a larger amount of digestion of milk-casein, and particularly in the class of amido compounds.

(3) In the case of the rennet-extracts in neutral solution, we notice that there was a small, but noticeable increase of water-soluble nitrogen compounds not observed in the case of pure pepsin. This increase was confined mostly to the caseoses and peptones. This increase may be due to the presence of some proteolytic enzyme, able to act in neutral solution, present in the rennet-extracts besides the rennet-enzyme proper. Granting that there is regularly present such an extra enzyme in rennet-extract, it could have very little to do with cheese-ripening, since in our experiments with milk we had much larger proportions of rennet-extract than are used in cheese-making. We added to the milk about 14 times as much rennet-extract as we commonly use in cheese-making; and this amount remained in the milk all the time, while in cheese-making some of the rennet-ferment passes into the whey.

(4) The increased activity of rennet-extract as well as of pepsin in the presence of acid is very marked.

EXPERIMENTS IN THE USE OF COMMERCIAL PEPSIN IN CHEESE-RIPENING.

In experiments 55, 56 and 57, the cheeses were made without

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chloroform in the normal way, except that the milk was pasteurized at 85° C. (185° F.) and hydrochloric acid was used in the place of lactic acid or a "starter." In 55, rennet-extract alone was used at the usual rate of 2.5 ounces for 1,000 pounds of milk. In 56, in addition to rennet-extract, we added 1 gram of Parke, Davis & Co.'s aseptic scale pepsin dissolved in water, and in 57, we used 15 grams of the pepsin and the usual amount of rennet-extract. We began to add the hydrochloric acid when the milk was at 29.5° C. (85° F.), the additions being made in quantities of 5 cc. to 20 cc. at intervals, until the milk coagulated in 30 seconds by the Monrad test. After the curd was cut, we added portions of 20 cc. of hydrochloric acid at intervals of 5 to 15 minutes. being guided by the general behavior of the curd. No salt was added to the curd. In other respects, the method of manufacture was normal. The cheeses were ripened at 15.5° C. (60° F.), and were analyzed at intervals. The detailed results of the chemical work can be found in the Appendix. In Table IV, we give the analytical results found in the fresh cheese and at the time of the last analysis.

TABLE IV.—SHOWING EFFECT OF COMMERCIAL PEPSIN IN CHEESE-RIPENING.

No. of experiment.	Age of cheese when analyzed.	Enzymes added.	Nitrogen, expressed as percentage of nitrogen in cheese, in form of—				
			Water-soluble nitrogen compounds.	Paracasein monolactate.	Paranuclein, caseoses and peptones.	Amides.	Ammonia.
			Per ct.	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*
55	Fresh	Rennet extract.	4.76	65.45	2.41	2.36	0
55	6 mos.		28.37	17.14	15.87	6.35	2.00
56	Fresh	Rennet and 1 gr. pepsin.	6.97	36.76	4.11	2.86	0
56	6 mos.		29.80	17.04	16.47	7.10	1.91
57	Fresh	Rennet and 15 gr. pepsin.	25.00	59.53	22.80	2.20	0
57	3 mos.		46.67	11.61	41.00	5.68	0.49

In studying the results contained in Table IV, we notice:

(1) The use of 1 gram of commercial pepsin in addition to

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rennet-extract slightly increased the proteolytic results in the cheese. This cheese contained considerably less moisture that 55 or 57.

(2) The use of 15 grams of commercial pepsin along with rennet-extract produced very marked results. This is strikingly evident in the fresh cheese, where we have 25 per ct. of the nitrogen in the cheese present in the form of water-soluble compounds, while in the case of experiment 55, in which rennet-extract only was used, the amount of soluble nitrogen compounds is less than 5 per ct. At the end of 3 months, we still have much more of the soluble nitrogen compounds in 57, the pepsin cheese, than we have in 55, the rennet-extract cheese, at the end of 6 months.

(3) In comparing the proteolytic factors in experiments 55 and 57, the conditions of work were such that the chief essential difference was the presence of pepsin in the latter, though 57 contained more moisture than 55. The observed difference in the chemical results could, therefore, be due only to pepsin, and this would be particularly true of the results obtained in the fresh cheese.

ACTION OF RENNET-ENZYME AND COMMERCIAL PEPSIN ON PARACASEIN DILACTATE.

We have already given the results of our study relating to the action of rennet-enzyme and of commercial pepsin on casein in milk and on casein monolactate, in the presence of chloroform. We have studied the action of these two enzymes also on the proteids of cheese made from pasteurized milk. We will now present the results of some work done in studying the action of these same enzymes on paracasein dilactate. Paracasein monolactate was extracted from several pounds of cheese by a 10 per ct. solution of sodium chloride and this was treated with acid, precipitating paracasein dilactate. Of this compound washed free from salt, we placed 25 grams, suspended in water, in each of several flasks and sterilized by heat. We then sterilized some solution of pepsin and rennet-extract by treating with 0.5 per ct. of formalin, containing 0.2 per ct. of formaldehyde. Accord-

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ing to Bliss and Novy,[4] pepsin is not affected by a 1 per ct. solution of formaldehyde nor rennet by a 4 per ct. solution. In one set of flasks, we added to each 0.06 gram of the sterilized pepsin, and in each of the other set of flasks 0.5 cc. of the sterilized rennet-extract. Duplicates were used in all cases. These were examined bacteriologically and chemically at intervals for 3 months. The formalin was very effective in destroying bacterial forms. In some cases a few molds were found, but not in sufficient number to affect the work. The nitrogen in the material was 4.35 per ct.

TABLE V.—SHOWING EFFECT OF RENNET-ENZYME AND COMMERCIAL PEPSIN ON PARACASEIN DILACTATE.

Enzymes used.	Age when analyzed.	Nitrogen, expressed as percentage of nitrogen in mixture, in form of—				
		Water-soluble nitrogen compounds.	Paracasein monolactate.	Paranuclein caseoses and peptones.	Amides.	Ammonia.
		Per ct.	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*
Pepsin	2 weeks	33 68	2.30	.....	.....	0
Rennet	" "	34.95	2.30	.....	.....	0
Pepsin	1 mo.	41.61	.....	37.87	3.74	0
Rennet	" "	43.68	.....	40.00	3.68	0
Pepsin	3 "	55.75	.....	46.55	9.20	0
Rennet	" "	57.25	.....	49.53	7 72	0

From the data contained in Table V, we can see that the results of our work indicate that:

(1) Both pepsin and rennet-enzyme exerted a marked proteolytic effect upon the paracasein dilactate, digesting about one-third of it in 2 weeks and considerably over one-half in 3 months. While the rennet-enzyme appears somewhat more active in forming water-soluble nitrogen compounds, the actual difference is small.

(2) Both enzymes formed amides in small quantities, but neither produced any ammonia.

(3) If we compare the results in Table V with those in Table III, we find that at the end of 1 and 3 months, more proteolysis occurred in this experiment than in the presence of chloroform. This is true of both enzymes. This suggests that the chloroform

[4] *Jour. Experimental Med.*, **4**: No. 1 (1899)

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may exert a retarding influence upon the action of pepsin and rennet. Malfitano[5] makes the statement that the action of pepsin is considerably diminished by chloroform. The difference noted in our work may be due to the greater amount of acid present in the experiment in Table V. However, both sets of experiments practically agree in showing small formation of amides and entire absence of ammonia.

EXPERIMENTS IN MAKING CHEESE FROM PASTEURIZED MILK WITH "STARTER."

For the purpose of comparison, it was regarded as desirable to have some cheeses made from pasteurized milk. The cheeses in experiments 52 and 53 were made for this purpose. We pasteurized 135 pounds of milk at 85° C. (185° F.), cooled it to 29° C. (84° F.), passed carbon dioxide gas through it for half an hour, introduced 4.5 pounds of a specially prepared lactic acid "starter," added Hansen's rennet-extract at the rate of 2.5 ounces for 1,000 pounds of milk, and then carried on the operation of cheese-making in the usual way. The curd was divided into two equal parts and one part, unsalted, was made into cheese No. 52, while the other portion, salted at the usual rate, was made into No. 53. Both cheeses were ripened at a temperature of 15.5° C. (60° F.).

As factors active in causing proteolytic changes, we had in the cheeses made in these two experiments (1) acid, (2) rennet-enzyme and (3) such micro-organisms as happened to be introduced with the "starter" and from the air of the room. As compared with a normal cheese, there were no milk-enzymes present and the biological factor would be expected to be considerably less marked. In comparison with the cheeses made in experiments 44 to 51, we had in 52 and 53 no chloroform, a difference that meant absence of a biological factor in the former case. In 52 and 53 the acid was furnished by a "starter," while in the other experiments artificial acid was added. In Table VI we give the results of chemical analysis made when the cheese was fresh from press and when 9 months old. The detailed analyses are given in the Appendix.

[5] *Ann. Inst. Pasteur*, **16**: 853 (1902).

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TABLE VI.—SHOWING COMPOSITION OF CHEESE MADE FROM PASTEURIZED MILK.

No. of experiment.	Age of cheese when analyzed.	Nitrogen, expressed in percentage of nitrogen in cheese, in form of—				
		Water-soluble nitrogen compounds.	Paracasein monolactate.	Paranuclein, caseoses and peptones.	Amides.	Ammonia.
52	Fresh	2.92	12.83	2.92	0	0
52	9 mos.	28.87	5.57	13.20	15.67	1.44
53	Fresh.	3 33	9.88	3.33	0	0
53	9 mos.	22.20	4.71	12.38	9.82	1.18

In studying these results, we notice:

(1) There was an increase in all the different classes of water-soluble compounds during the 9 months of ripening.

(2) The amount of amido compounds was considerably in excess of the amounts found in cheese made with chloroform.

(3) Ammonia was formed in 52 and 53, while none was present in experiments 44 to 51.

(4) The increased amount of amido compounds and of ammonia observed in experiments 52 and 53, as compared with experiments 44 to 51, must be ascribed to the presence in the former of an active biological factor.

THE DESTRUCTION OF MILK-ENZYMES BY HEAT.

In our experiments in using pasteurized milk for studying the proteolytic action of rennet-enzyme in cheese and milk, we have stated that no enzyme was present in the milk when made into cheese except that added in the rennet-extract. It is well known that milk contains a proteolytic enzyme, as shown first by Babcock and Russell and confirmed by our own work and that of others. In studying the action of rennet-enzyme, it is essential that all other enzymes previously existing in the milk shall be rendered inactive. It is commonly held that these enzymes are destroyed at 85° C. (185° F.). After pasteurizing the milk used in our various experiments, we took the precaution to keep samples of the milk for examination. These samples were treated with 3.5 per ct. of chloroform by volume and determinations of the soluble nitrogen compounds were made at intervals. The results of this work are given in the accompanying table.

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TABLE VII—SHOWING EFFECT OF HEAT ON PROTEOLYTIC ENZYMES IN MILK.

No. of experiment.	Temperature used in heating milk.	Age of milk when analyzed.	Soluble nitrogen expressed in percentage of nitrogen in milk.
	Degrees	*Months*	*Per ct.*
General test	90°C.(194°F.)	13	4.26
" "	85°C.(185°F.)	8	10.8
" "	85°C.(185°F.)	7	9.7
45 and 46	95°C.(203°F.)	15	5.52
47	95°C.(203°F.)	16	5.5
51	98°C.(208°F.)	14	11.5

We have found that the percentage of soluble nitrogen in nearly fresh milk is often as high as 10 per ct. of the nitrogen in the milk. These results show that, during the long period of time indicated, no proteolysis had occurred and that we are justified in saying the milk was enzyme-free after heating.

EFFECT OF COMMON SALT ON ACTION OF RENNET IN CHEESE-RIPENING.

In Bulletin No. 203, page 241, we gave results showing that salt, in the proportion of about 1 per ct., the amount usually present in cheese, exerts a rather marked repressing influence upon the proteolytic action of those enzymes that are present in milk when made into cheese. We have also found that, in normal cheese, the addition of increased quantities of salt decreases the rapidity of proteolytic action. Some of our experiments were planned with a view to study the action of salt on cheese-ripening when rennet-enzyme is the only proteolytic factor present. In experiments 44 and 47, the results were negative because, in the absence of acid, no ripening change of any kind occurred. In experiments 45 and 46, the amount of soluble nitrogen was the same with and without salt, but was rather small in both cases, compared with normal cheese. In experiments 48 and 51, larger amounts of soluble nitrogen compounds were formed in the presence of salt. This may have been in part due to the fact that cheese 48 contained much moisture and was kept at a little higher temperature for the first few weeks. In experiments 52 and 53, the formation of soluble nitrogen compounds was less when salt was added; but, in these cases, we had present biological

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factors not found in the other experiments. So far as these results go, they appear to indicate that, in cheese-ripening, salt, in the proportions commonly used, has little or no influence upon the action of rennet-enzyme, and that the retarding action observed in normal cheese, due to salt, comes from its influence upon other proteolytic agents. The results appear to us to call for additional work, before this point can be regarded as definitely settled. It may be mentioned in this connection that Chittenden and Allen[6] have shown that the action of pepsin in digesting blood-fibrin is diminished by the presence of common salt.

EFFECT OF ABNORMAL CONDITIONS PRESENT IN EXPERIMENTS.

We have already called attention to the difference of conditions present in the experiments described in this bulletin and those found in normal cheese. We will now consider these in more detail. These abnormal conditions found in our experiments, but not present in normal cheese, are the following: (1) Milk heated to 85° C. to 98° C. (185° F. to 208° F.) to destroy all enzymes originally existing in milk; (2) the use of calcium chloride or carbon dioxide gas to restore the coagulating property of milk-casein by rennet-extract; and (3) the use of chloroform to suppress all activity of organisms. The question naturally arises as to whether the introduction of these unusual conditions seriously affected the results obtained and, if so, in what manner and to what extent.

Does the pasteurizing of milk affect the proteolytic action of rennet-extract in relation to cheese-ripening?—A study of the data embodied in Tables I, II and III indicates that when the conditions were favorable for the action of rennet-enzyme or pepsin, we found more or less proteolysis taking place in cheese made from milk that had been heated as high as 98° C. (208° F.). In experiments 44, 47, 49 and 50, our results were negative, not because the milk had been heated, but because no acid was present, a condition that is essential for the action of rennet-ferment. In experiments 45, 46, 48 and 51, varying degrees of proteolysis were found but in these experiments acid was present, the milk

[6] Studies in Physiol. Chem. Yale Univ. 1 : 92 (1884-85).

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having been heated as in the other cases. While we are unable, from any data known to us, to say whether rennet-enzyme would act any more vigorously in the case of cheese made from milk that had not been heated, we can say that the heating of milk does not prevent proteolysis, though possibly it may retard it somewhat, a point upon which we have no positive evidence. The fact that heating milk above a certain temperature weakens the action of rennet-enzyme in coagulating milk-casein may or may not be suggestive that the proteolytic function of rennet-ferment is also affected unfavorably. The vigorous digesting action of rennet-extract and of commercial pepsin on the casein of milk heated to 85° C. (185° F.) suggests that heat does not seriously affect the proteolytic action of rennet-enzyme; but the results of this experiment are not strictly applicable to results obtained with cheese, because we had much larger quantities of rennet-enzyme working in the milk than we had in the case of cheese.

Effect of calcium chloride and of carbon dioxide gas on the proteolytic action of rennet-extract in cheese-ripening.—In making a study of the series of experiments in which calcium chloride was used (44 to 47), we found that little or no digestion was taking place. It occurred to us that possibly this salt might have some repressing influence upon enzyme action. We then made a parallel series of experiments (48 to 51), in which the use of calcium chloride was replaced by carbon-dioxide gas. In studying our results, we are unable to reach any definite conclusion in regard to the action of calcium chloride. Additional work is needed to settle this point definitely.

The use of calcium chloride is more convenient than that of carbon dioxide gas, but the latter is preferable in the following respects: (1) We obtain a curd more nearly normal in its general physical properties when carbon dioxide is used; (2) any excess of carbon dioxide is easily removed; (3) carbon dioxide is less likely to introduce permanently any abnormal chemical and biological conditions than is calcium chloride. So far as our results indicate, carbon dioxide by itself has no power to form with paracasein any salt-soluble compounds. This is shown particularly by experiment 49, Table I, in which neither acid nor salt was used and in which there was found increase of neither water-soluble nor salt-soluble compounds.

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Effect of chloroform on the action of rennet-enzyme in cheese-ripening.—We have already called attention to the point that a comparison of the results contained in Tables III and V suggests that chloroform may exert some retarding influence upon the action of rennet-enzyme in cheese-ripening. In Bulletin No. 203, page 224, we published some results which appeared to indicate that chloroform has little or no effect upon galactase, but those results do not necessarily apply to any other enzyme. The work of Malfitano, already referred to, indicates that the action of a peptic ferment is retarded by chloroform.

DISCUSSION OF RESULTS.

In the work described in the preceding pages, we have studied the proteolytic action of rennet-enzyme under the following conditions:

(1) *In cheese containing rennet-enzyme as the only proteolytic agent, with and without acid, and also with and without salt.*—In these experiments (44 to 51), all milk-enzymes were destroyed by heating at 95° C. to 98° C. (203° F. to 208° F.), the coagulable property of the milk-casein was restored by the addition of either calcium chloride or carbon dioxide gas, and all organisms were rendered inactive by chloroform. Acid, when present, was furnished by addition of pure lactic acid.

(2) *In cheese containing rennet-enzyme together with acid-forming and some proteolytic organisms.*—In these experiments (52 and 53), the milk enzymes were destroyed by heating, acid was furnished by a lactic-acid "starter," but no chloroform was used. We thus had, as our only proteolytic agents, rennet-enzyme in the presence of acid and some liquefying organisms that were introduced in the "starter" or that got into the milk or curd during the operation of cheese-making.

(3) *In cheese containing commercial pepsin in addition to rennet-enzyme, together with hydrochloric acid and such organisms as were introduced during the process of making cheese.*—In these experiments (55 to 57), the milk enzymes were destroyed by heat and commercial pepsin added in different amounts.

(4) *In comparison with commercial pepsin on casein in milk, with and without acid.*—In these experiments, the milk-enzymes were

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destroyed by heat and all organisms were rendered inactive by chloroform.

(5) *In comparison with commercial pepsin on paracasein dilactate.* —In these experiments, rennet-enzyme and commercial pepsin, sterilized by formaldehyde, were allowed to act upon sterile paracasein dilactate.

The results of these experiments appear to us to justify the following statements:

(1) In the case of every experiment made, whether with cheese or milk, there was little or no proteolytic action of either rennet-enzyme or commercial pepsin in the absence of acid; while there was marked action, though in varying degrees, in the presence of acid.

(2) In the absence of acid in cheese, no paracasein lactate is formed and little or no proteolysis occurs; in the presence of acid in cheese, or more strictly in the milk and curd, paracasein monolactate is formed and proteolysis takes place, with the rennet-ferment as the active agent. The ability of rennet-enzyme to convert paracasein into soluble nitrogen compounds appears to depend upon the presence of paracasin lactate. In cheese-making, therefore, the primary function of acid appears to be the formation of a chemical compound with paracasein, commonly paracasein monolactate but, in excess of acid, paracasein dilactate. The conversion of parcasein monolactate by rennet-enzyme into soluble nitrogen compounds is strongly suggested by the fact that, when the soluble nitrogen compounds increase, the paracasein monolactate decreases.

(3) In comparing rennet-enzyme and commercial pepsin in the case of cheese, milk and paracasein dilactate, the experiments that were strictly parallel have shown about the same extent of proteolytic action.

(4) In the case of both rennet-enzyme and commercial pepsin, the chemical work performed by the ferments is confined mainly to the formation of paranuclein, caseoses and peptones, while only small amounts of amides are formed, and no ammonia.

(5) Rennet-enzyme is a peptic ferment, as shown by the following characteristics: (*a*) neither rennet-enzyme nor pepsin causes much, if any, proteolytic change, except with the help of acid; (*b*) the quantitive results of proteolysis furnished by rennet-

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enzyme and pepsin agree closely when working on the same material under comparable conditions; (*c*) the classes of soluble nitrogen compounds formed by the two enzymes are the same both qualitatively and quantitatively; (*d*) neither enzyme forms any considerable amount of amido compounds, and neither produces any ammonia; (*e*) the soluble nitrogen compounds formed by either enzyme are chiefly confined to the groups of compounds known as paranuclein, caseoses and peptones.

(6) The experiments made to determine the influence of salt on the proteolytic action of rennet-enzyme, while not conclusive, suggest that salt has little or no effect upon the action of rennet-enzyme in cheese-ripening.

(7) In obtaining our results relating to the study of the function of rennet-enzyme in cheese-ripening, we were necessarily compelled to work under conditions more or less abnormal as compared with the conditions commonly present in cheese-making. The effect of such unusual conditions would tend, if they had influence at all, to diminish the proteolytic action of rennet-enzyme. We are, therefore, justified in believing that our results represent the minimum effect of rennet-enzyme in cheese-ripening and that, under normal conditions, it takes, if anything, a larger part than that indicated by our experiments.

(8) In some experiments, we eliminated all milk-enzymes and all active forms of organisms contained in the milk before making it into cheese. In some cases, we had rennet-enzyme in the presence of acid as the only proteolytic agent in the cheese; in others, we had the same conditions and, in addition, such proteolytic organisms as chanced to get into the milk and curd during the process of cheese-making. In the latter case (52 and 53), larger amounts of amides were formed, and some ammonia; while, in the presence of rennet-enzyme alone, no ammonia was formed and only small amounts of amido compounds. When we compare normal cheese with cheese containing only rennet-enzyme, we find the same difference, except that it is more pronounced, as we should expect. Hence, the special work done by the rennet-enzyme as a factor in cheese-ripening is that of a peptic digestion, forming groups of water-soluble nitrogen compounds, intermediate in complexity of structure between paracasein and the amido compounds, viz., paranuclein, caseoses and peptones.

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In normal cheese, we find an accumulation of amides and ammonia, as the cheese grows older and a corresponding diminution of the compounds previously formed. The formation of all the ammonia and of a large proportion of the amides found in ripened cheese must be due to some agency other than rennet-enzyme, and the only other agents present, besides milk-enzymes, that can do this work appear to be organisms or their enzymes. The first stage in normal cheese-ripening is essentially a peptic digestion of paracasein monolactate. Gradually amides are formed and later ammonia. It is probable that the first chemical work done in normal cheese-ripening is the conversion of paracasein monolactate by rennet-enzyme into paranuclein, caseoses and peptones. The question naturally arises as to whether these compounds must be formed before other agents can take part in the work and carry it along farther, producing amides and ammonia. We are at present engaged in studying this phase of the problem.

9. When rennet-enzyme was the only digesting agent in cheese, we were unable in any case to find the slightest traces of cheese flavor. Apparently, we must look to other sources for this important product of cheese-ripening.

APPENDIX.

It has been considered desirable to present in greater detail the data relating to the conditions of the experiments and to the analytical results, in order that those who are especially interested in the work may have access to these details.

CONDITIONS OF EXPERIMENTS IN CHEESE-MAKING.

In experiments 44 to 53 and 55 to 57, rennet was used at the uniform rate of 2.5 ounces for 1000 pounds of milk, and salt, when added, was used at the rate of 2 pounds for 1000 pounds of milk. In all experiments, the usual conditions of manufacture were followed as closely as possible. Chloroform, when used, was added in quantities to equal 3 to 5 per ct. of the milk by volume. The cheeses were in most cases cured at 15.5° C. (60° F.). We give in the following table the other details. The + sign shows that a certain condition was present, while the o sign shows that the condition in question was not present.

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CONDITIONS OF MANUFACTURE OF EXPERIMENTAL CHEESES.

No. of experiment.	Date of making cheese.	Chloroform used.	Cheese salted.	Kind of acid used.	Milk heated.	Calcium chloride used.	Carbon dioxide used.	Pepsin used.	Pounds of milk used.
					Temp.				
44	Nov. 11, 1901	+	o	o	98° C	+	o	o	50
45	" 21, "	+	o	Lactic	"	+	o	o	125
46	" 22, "	+	+	Lactic	"	+	o	o	
47	" 23, "	+	+	o	"	+	o	o	50
48	Dec. 24, "	+	+	Lactic	"	o	+	o	40
49	" 24, "	+	o	o	"	o	+	o	75
50	Jan. 4, 1902	+	+	o	"	o	+	o	
51	" 7, "	+	o	Lactic	"	o	+	o	40
52	" 13, "	o	o	Starter	"	o	+	o	135
53	" 14, "	o	+	Starter	"	o	+	o	
55	July 9, "	o	o	Hydrochloric	85°	o	o	o	286
56	" 11, "	o	o	Hydrochloric	"	o	o	1 gram	279
57	" 13, "	o	o	Hydrochloric	"	o	o	15 grams	280

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DETAILS OF CHEMICAL ANALYSES.

The methods of analysis employed are those fully described in Bulletin No. 215 of this Station. Even with the exercise of extreme precaution, it is difficult always to secure from the same cheese samples that will give uniform analytical results, since different portions of a cheese may vary in composition. Such inconsistencies as appear in different analyses of the same cheese are to be attributed largely to variations of different samples. At the same time, it should be remembered that our methods of separation are far from perfect. In some cases, we give the results secured for amido compounds with both reagents, phosphotungstic acid and tannic acid. In peptic digestions, we regard the determination of amides by phosphotungstic acid as being much nearer the actual truth.

EXPERIMENT 44.

Age of cheese when analyzed.	Per ct. of water in cheese.	Per ct. of chloroform in cheese.	Per ct. of nitrogen in cheese.	Nitrogen, expressed as percentage of nitrogen in cheese, in form of —					
				Paracasein monolactate.	Total water-soluble nitrogen compounds.	Paranuclein, caseoses and peptones.	Amides by phosphotungstic acid.	Amides by tannic acid.	Ammonia.
				Per ct.	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*
Fresh	47.00	5.9	3.11	2.44	1.93	1.93	.....	0	0
1 week	47.70	6.1	3.18	2.52	4.40	3.14	.....	1.26	0
2 "	46.00	5.9	3.11	2.25	3.34	2.28	.....	0.96	0
1 month	46.70	6 1	3.13	2 43	4.22	4.22	.....	0	0
2 "	44.60	8 8	3.24	2.72	3.77	3.77	.....	0	0
6 "	47.50	6.4	3.30	3.39	7.39	3 46	.....	3 94	0
9 "	46 50	7.2	3.39	2.36	6 84	2.71	2 95	4.13	0
12 "	45.20	7 0	3.68	2.72	6 25	2.96	3.29	3.29	0

EXPERIMENT 45.

Fresh	41.65	10.6	3.12	27.88	4.65	3 37	.....	1 28	0
1 Week	42.00	10.4	3.13	27.16	3.83	3 83	.....	0	0
1 Month	42.25	9 6	3.12	22 24	7.57	4.68	.....	2.89	0
2 "	39.25	12.4	3.15	16.29	11.30	10 67	.....	0.63	0
6 "	41.50	11.0	3.24	18.06	17.53	14.14	.....	3.39	0
9 "	40.50	12.0	3.32	19.64	16.15	13.74	2 41	7.53	0
12 "	40.00	12.0	3.57	9.80	18.50	14.38	4.12	4.90	0

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EXPERIMENT 46

Age of cheese when analyzed.	Per ct. of water in cheese.	Per ct. of chloroform in cheese.	Per ct. of nitrogen in cheese.	Nitrogen, expressed as percentage of nitrogen in cheese, in form of —					
				Paracasein monolactate.	Total water-soluble nitrogen compounds	Paranuclein, caseoses and peptones.	Amides by phosphotungstic acid.	Amides by tannic acid.	Ammonia.
				Per ct.	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*
Fresh	47.70	9.6	2.78	26.62	5.40	3.96	.....	1.44	0
1 Week	46.60	9.8	2.79	25.09	3.44	3.44	.....	0	0
1 Month	44.40	9.6	2.84	22.19	9.71	8.16	.....	1.55	0
2 "	43.90	11.6	2.89	28.52	11.66	11.21	.....	0.45	0
6 "	46.00	10.4	2.97	19.06	18.90	14.72	.....	4.21	0
9 "	44 00	10.8	3.04	19 94	17.17	15.52	1.65	6.58	0
12 "	.....	.....	3.35	11 97	18.50	14.02	4.48	4.48	0

EXPERIMENT 47.

Fresh	48 50	9 8	2.62	2.90	3.67	3.67	.....	0	0
1 week	47.40	10 4	2.72	2.21	3.60	3 60	.....	0	0
1 month	47 40	10.2	2.67	2.10	4.00	2.88	.....	1.12	0
2 "	47.30	.....	2.70	3.12	5.78	5.41	.....	0.37	0
6 "	47.00	11.0	2.77	3.03	8 81	5.20	.....	3.61	0
9 "	46.00	11.6	2 84	2.47	5.99	3 88	2.11	3.35	0
12 "	.....	.....	3.12	3.46	6.41	3.08	3.33	3 52	0

EXPERIMENT 48.

Fresh	53 00	8.9	2.35	29.80	4.26	3.41	.....	0.85	0
2 weeks	51.40	11.2	2.30	27.40	13.04	11.91	.....	1.13	0
1 month	50.00	10 2	2.51	27.09	13.95	11.20	.....	1.75	0
3 "	50.54	12.0	2.41	27.22	32.37	29.88	.....	2 49	0
6 "	49.20	13.2	2.48	25 41	27.99	24.36	.....	3.63	0
9 "	49.50	13.0	2.48	20.97	36 30	32.67	.....	3.63	0
12 "	.....	.....	2.62	12.98	40.84	35.88	4.96	6.30	0
15 "	.....	.....	2.55	11.76	47.06	40.00	7.06	9.40	0

EXPERIMENT 49.

Fresh	55.60	6 6	2.52	5.24	10.95	9.92	.....	1.03	0
2 weeks	55.00	6.4	2.61	4.75	9.43	8 43	.....	1.00	0
1 month	54.20	5.8	2.64	2.54	5.76	5.76	.....	0	0
3 "	51.05	8.0	2.79	3.73	9.18	5.95	.....	3.23	0
6 "	49.40	8.5	3.12	2.56	10 00	7.11	.....	2.89	0
9 "	48.60	8.8	3.33	.....	7.81	4.61	3.20	3 90	0
12 "	.....	.....	3.28	4 27	10.34	5.16	5.18	4.57	0

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EXPERIMENT 50.

Age of cheese when analyzed.	Per ct. of water in cheese.	Per ct. of chloroform in cheese.	Per ct. of nitrogen in cheese.	Nitrogen, expressed as percentage of nitrogen in cheese, in form of —					
				Paracasein monolactate.	Total water-soluble nitrogen compounds	Paranuclein caseoses and peptones.	Amides by phosphotungstic acid.	Amides by tannic acid.	Ammonia.
				Per ct.	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*
Fresh	54.50	6.7	2.59	5.72	10.12	8.57	.....	1.55	0
2 weeks	55.00	6.5	2.55	5.10	9.49	7.53	.....	1.96	0
1 month	52.50	8.4	2.52	3.97	6.35	6 35	.....	0	0
3 "	50.30	9.6	2.79	3.16	9.90	6.30	.....	3 60	0
6 "	51.30	9.0	3.06	3.14	12.75	9.81	.....	2.94	0
9 "	51.50	9.0	3.20	2.81	7.50	3.75	3.75	3.75	0
12 "	.....	.....	3.31	3.02	9.67	5.14	4.53	5.14	0

EXPERIMENT 51.

Fresh	38.15	13.2	3 29	22.92	3.89	3.11	.....	0.79	0
2 weeks	38.60	13.2	3.32	23.68	5.66	5 03	.....	0.63	0
1 month	37.30	16 0	3.10	22.97	5.30	5.30	.....	0	0
3 "	37.30	16.5	3.27	22.02	11.25	8.50	.....	2 75	0
6 "	39.00	15.8	3.27	17.62	12.48	10.95	.....	1.53	0
9 "	38.70	16.0	3.48	15.23	13.22	10.35	2 87	3.45	0
12 "	.....	.....	3.42	12.87	15.50	10.53	4.97	4.97	0
15 "	.....	.....	3.52	.....	19.32	15.06	4.26	5.12	0

EXPERIMENT 52.

Fresh	42.75	0	3.43	12.83	2.92	2.92	.....	0	0
2 weeks	39.20	0	3.68	11.68	7.45	4.73	.....	2.72	0.54
1 month	39.78	0	3.80	8.95	10 90	7.21	.....	3 69	0.68
3 "	35.10	0	4.08	8.78	17.21	8.21	.....	9 00	0.32
6 "	34.55	0	4.48	8.20	24.11	15.11	9.00	16.07	1.00
9 "	30.73	0	4.85	5.57	28.87	13.20	15.67	22.90	1.44

EXPERIMENT 53.

Fresh	45.73	0	3.24	9.88	3 33	3.33	.....	0	0
2 weeks	41.08	0	3.55	11.27	8.14	4 48	.....	3.66	0.56
1 mo.	41.52	0	3.61	8.80	11.08	6.65	.....	4.43	0 72
3 "	36.75	0	3.97	7.21	16.90	7.48	.....	9.42	0.15
6 "	36.41	0	4.49	3.15	20.72	13.61	7.11	12.47	1.11
9 "	27.10	0	5.09	4.71	22.20	12.38	9 82	16.30	1.18

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EXPERIMENT 55.

Age of cheese when analyzed.	Per ct. of water in cheese.	Per ct. of chloroform in cheese.	Per ct. of nitrogen in cheese.	Nitrogen, expressed as per centage of nitrogen in cheese, in form of —					
				Paracasein monolactate.	Total water-soluble nitrogen compounds	Paranuclein caseoses and peptones.	Amides by phosphotungstic acid.	Amides by tannic acid.	Ammonia.
				Per ct.	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*
Fresh	38.61	0	3.82	65.45	4.76	2.41	2.35	2.36	0
1 mo.	38.05	0	4.05	23.95	16.99	14.64	2.35	4.94	0.49
3 "	34.25	0	4.24	15.10	29.25	25.12	4.13	10.61	0.61
6 "	28.84	0	5.04	17.14	28.37	22.02	6.35	10.50	2.00

EXPERIMENT 56.

Fresh	33.75	0	4.19	36.76	6.97	4.11	....	2.86	0
1 mo.	31.82	0	4.48	23.22	18.08	16.29	1.79	6.92	0.54
3 "	28.31	0	4.64	15.09	26.08	21.34	4.74	11.21	0.56
6 "	24.30	0	5.07	17.04	29.80	22.70	7.10	11.42	1.91

EXPERIMENT 57.

Fresh	47.35	0	3.36	59.53	25.00	15.18	2.20	9.82	0
1 mo.	38.33	0	3.80	18.16	43.95	40.00	3.95	26.06	0.68
3 "	31.12	0	4.05	11.61	46.67	41.00	5.68	19.76	0.49

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